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TECHNICAL MANUSCRIPT 532

PRODUCTION OF ETHYLENE
BY PENICILLIUM CYCLOPIUM

Joseph Lonski
Harry E. Gahagan III

APRIL 1969

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TECHNICAL MANUSCRIPT 532

PRODUCTION OF ETHYLENE BY
PENICILLIUM CYCLOPIUM

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PLANT SCIENCES LABORATORIES

Project 1B562602A061

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ABSTRACT

Ethylene production by Penicillium cycloptum, ATCC No. 7615, was studied in relation to the life cycle of the fungus. Ethylene, >0.05ppm, was not produced until after the culture reached its maximum dry weight. When the sucrose concentration of the medium was varied, ethylene was proportional to growth. However, when the ammonium nitrate concentration was varied or the nitrogen source changed, there was no direct relationship. Inoculum size also influenced ethylene production. Ethanol markedly stimulated total ethylene production; dimedone was a good inhibitor of ethylene production.

The structure of the naturally produced ethylene was verified by converting it to 1,2-dibromoethane and showing that its infrared spectrum was identical to that of authentic 1,2-dibromoethane.

I. INTRODUCTION*

In 1951, Young, Pratt and Biale chemically identified a physiologically active gaseous emission of Penicillium digitatum as ethylene by preparation of chemical derivatives.¹ Gas chromatographic evidence and bioassays utilizing plant materials indicate that a number of other fungi may produce ethylene.²⁻⁴

We wish to report (i) that ethylene, identified by conversion to the 1,2-dibromoethane derivative, is a gaseous metabolite of Penicillium cycloptum, (ii) the relationship of the production of ethylene to the life cycle of the fungus, and (iii) some factors affecting the production of ethylene.

II. MATERIALS AND METHODS

The isolate of Penicillium cycloptum used in these studies was ATCC No. 7615 obtained from the American Type Culture Collection. Stock cultures were maintained on sterile potato-dextrose agar in the dark at 25°C. Inoculum was prepared from 7-day-old cultures by shaking the spores into a flask of sterile distilled water. A DeVilbiss atomizer No. 152, modified with a collar around the nozzle to prevent the escape of inoculum, was used to inoculate the flasks. Each flask contained approximately 250,000 spores. After inoculation the cultures were stoppered with cotton plugs. All cultures were grown in the dark at 25°C after preliminary experiments indicated no differences between dark- and light-grown cultures.

To study the relationship of ethylene to the life cycle of the fungus, the cultures were grown in 50-ml Erlenmeyer flasks on 5 ml of sterile medium. The basal culture medium had the following composition: sucrose, 2% g; NH_4NO_3 , 0.0 g; KH_2PO_4 , 11.6 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.23 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g; Difco agar, 8 g; 1.0 ml of microelement solution (supplied the following final concentrations of the elements per liter: Cu, 0.02 mg; Zn, 0.05 mg; Mo, 0.01 mg; Mn, 0.5 mg; and Bo, 0.5 mg); and redistilled water to make 1 liter. The pH of the medium was adjusted to 5.0 prior to autoclaving and was found to be 4.8 after autoclaving. This was in the optimum pH range for P. cycloptum.

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Every 8 hours the cotton plugs in each of five different flasks were replaced with sterile rubber vaccine stoppers. After 8 hours the ethylene, carbon dioxide, and oxygen content of the air space in the stoppered flasks was measured. Each value reported is the average of four replicates.

In the series of experiments in which ethylene production from various metabolites was studied, each value reported is the average of four replicates of three flasks each. Different 50-ml Erlenmeyer flasks were stoppered every 12 or 24 hours, and the ethylene and dry weight were measured.

The gases were measured with an F&M Scientific Corporation (Model 720) gas chromatograph.⁶ A flame ionization detector (hydrogen-oxygen flame) was used for ethylene and a thermal conductivity detector (175 ma) was used for carbon dioxide and oxygen. Columns used were molecular sieve for oxygen, silica gel for carbon dioxide, and activated alumina for ethylene.⁷

After the gas composition was determined, the flasks were warmed to melt the agar and the contents were filtered with suction on a Büchner funnel, using tared filter paper. The filtrate was retained for pH determination. The fungal mat was then washed with three 10-ml portions of hot distilled water. The filter papers and fungal mats were dried for 24 hours at 80 C and weighed to 0.1 mg.

To prepare the ethylene derivative 1,2-dibromoethane, cultures were grown in nine Fernbach flasks on 500 ml of sterile basal medium, supplemented with 4% asparagine per liter. After the cultures began producing ethylene, the cotton plugs were replaced with two-hole rubber stoppers arranged so that air could be swept through the flask. The air stream was passed through each flask for 30 minutes on four consecutive days, bubbled through 150 ml of 0.5% Br in CCl_4 , and finally bubbled through 100 ml of a 1% solution of NaSO_3 to trap any vaporized bromine. Then the CCl_4 was equilibrated with three 50-ml portions of 0.2 N NaOH to remove the unreacted bromine. After drying overnight with 20 g anhydrous CaSO_4 , the CCl_4 was reduced to a volume of 2 ml with a rotary flash evaporator and was reduced further under a stream of nitrogen.

The 1,2-dibromoethane synthesized from naturally produced ethylene and authentic 1,2-dibromoethane* were chromatographed with a Perkin-Elmer gas chromatograph equipped with a flame ionization detector (hydrogen-air flame) and stream splitter.

Samples were trapped in 0.01 mm path-length AgCl infrared cells, using a heated collection line (RIIK) and a Dry Ice ethyl alcohol trap.

Infrared spectra were run on a Beckman IR8.

* Fisher Chemical Co., Silver Spring, Md.

III. RESULTS

A. IDENTIFICATION OF ETHYLENE

The retention time of the gas produced by P. cyclopium was identical to that of commercial ethylene. The brominated derivative had a retention time identical to that of commercial 1,2-dibromoethane and had an identical infrared spectrum.

B. RELATIONSHIP OF THE PRODUCTION OF ETHYLENE TO THE LIFE CYCLE

Penicillium cyclopium grows rapidly under the previously described cultural conditions (Fig. 1), although the time required to reach maximum mycelial mass varies with the amount of inoculum (Fig. 2). When the basal medium was used, growth preceded ethylene production by more than 60 hours. Under all experimental conditions studied, ethylene was produced only after the growth had passed its maximum, as determined by dry weight. The cessation of growth was probably caused by lack of carbohydrate, as additional carbohydrate supplied at the time of inoculation resulted in greater dry weights (Table 1). The loss in dry weight probably indicates the onset of autolysis.

Microscopic examination of the cultures verified that ethylene production is initiated after the onset of sporulation. The appearance of the cultures also changed from white to olive green prior to ethylene evolution.

Preliminary experiments indicated that the optimum pH of the medium for both growth and ethylene production is 4.8 at the time of inoculation. Figure 1 shows that the pH of the medium falls to about 3.6 during the log phase of growth and then gradually rises to about 5.9 during the initiation of ethylene production and probable onset of autolysis.

The respiratory quotient (RQ) was computed from the oxygen consumption and carbon dioxide production for each 8-hour period. The RQ reaches a maximum of about 1.3 during the period of maximum growth and falls below 1.0 soon after the culture begins to lose weight.

Figure 1 shows that no measurable ethylene (>0.05 ppm) is recorded until the growth rate has passed its maximum. This was true under all experimental conditions studied.

C. RELATIONSHIP OF SUCROSE TO ETHYLENE PRODUCTION

Table 1 shows the effect of varying the sucrose concentration in the basal medium on total dry weight and ethylene production. When the sucrose concentrations were varied, the growth (mg dry wt) was proportional to the sucrose concentration, and total ethylene production was proportional to growth. When the ethylene production is converted to microliters per gram dry weight, there is no significant difference among the four sucrose concentrations tested.

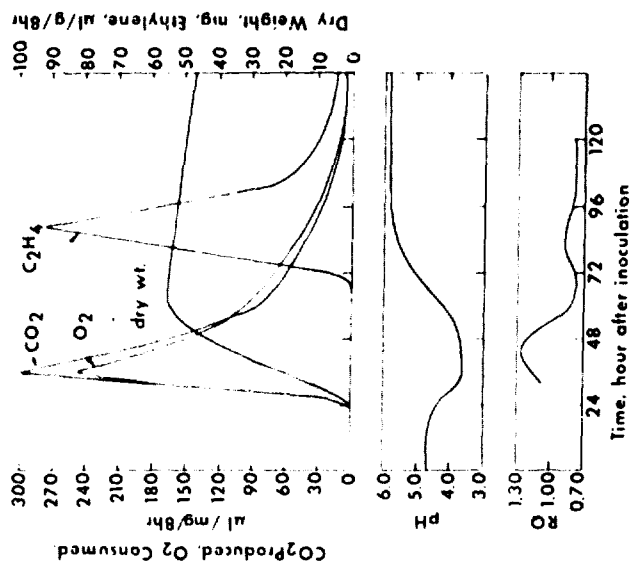


FIGURE 1. Growth, O_2 Consumption, CO_2 and C_2H_4 Production of *P. cyclospium* Grown on the Basal Medium at $25\pm 2^\circ C$ for 6 Days. Every 8 hours five different flasks were sealed and the three gases, pH , and dry weight measured after 8 hours. The respiratory quotient was derived from the O_2 and CO_2 values recorded every 8 hours on the chromatograph. Each value represents the average of four replicates.

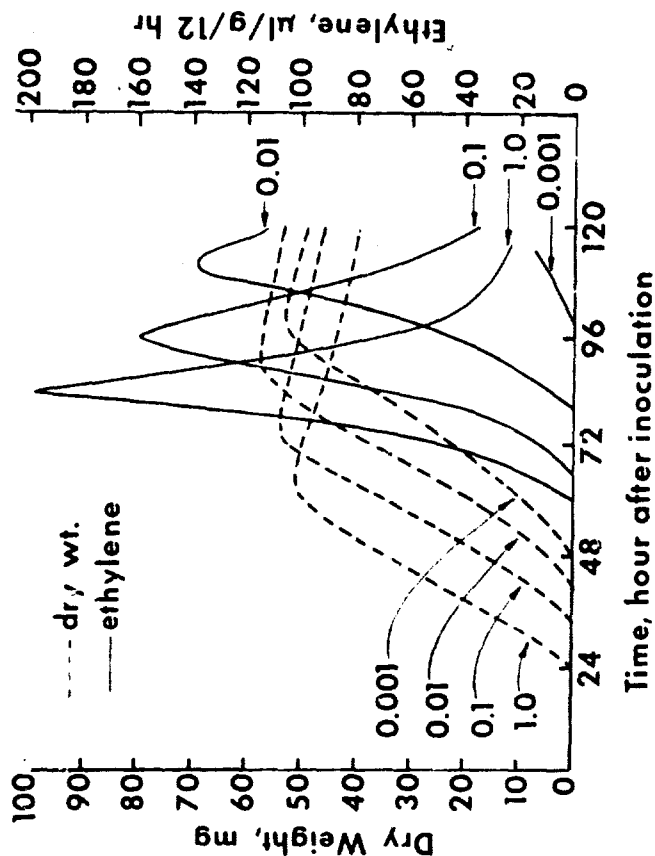


FIGURE 2. Effect of Inoculum Concentration on Growth and C_2H_4 . The highest concentration contained approximately 500,000 spores per ml; this was diluted with sterile distilled water to yield additional concentrations of 0.1, 0.01, and 0.001% of this value. The inoculum, 0.5 ml, was applied to sterile basal medium and grown at $25\pm 2^\circ C$ for 6 days. C_2H_4 and dry weight were determined every 12 hours. Each value represents the average of four replicates of three flasks each.

TABLE 1. EFFECT OF SUCROSE CONCENTRATION ON ETHYLENE PRODUCTION^a/

Sucrose, grams/liter	Dry Weight, mg	Ethylene, μliter/g dry wt	Time, hours
5	10	145	78-90
10	19	152	78-90
15	29	144	78-90
25	43	155	90-102

- a. The sucrose concentration of the basal medium was varied. Cultures were followed for 9 days and sampled for ethylene and dry weight every 12 hours. The reported values are the maximum recorded during the 9 days. Time refers to maximum ethylene production with maximum dry weight occurring prior to this. Each value represents the average of four replicates of three flasks each.

D. RELATIONSHIP OF AMMONIUM NITRATE TO ETHYLENE PRODUCTION

Table 2 shows the effect of varying the ammonium nitrate concentration in the basal medium on total dry weight and ethylene production. Except for the highest concentration, there is no significant difference in dry weights. Ethylene production was not proportional to growth. Maximum ethylene production was obtained with 2 grams per liter and good ethylene production from both 4 and 8 grams per liter.

E. RELATIONSHIP OF NITROGEN SOURCE TO ETHYLENE PRODUCTION

Figure 3 shows the utilization of different nitrogen compounds by *P. cyclopium* over a 9-day period. The nitrogen compounds were substituted singly in the basal medium in amounts to give 1.4 grams of nitrogen per liter. Each nitrogen source tested was successfully utilized by the fungus for growth. Growth did not vary appreciably but the ethylene production appears to be dependent on the nitrogen source, and not proportional to growth. Ammonium nitrate, L-asparagine, and ammonium citrate yielded the greatest amount of ethylene.

TABLE 2. EFFECT OF AMMONIUM NITRATE CONCENTRATION ON ETHYLENE PRODUCTION^a

NH_4NO_3 , grams/liter	Dry Weight, mg	Ethylene, $\mu\text{liter/g dry wt}$	Time, hours
1	47	22	114-126
2	50	172	90-102
4	48	146	90-102
8	45	124	90-102
16	36	21	90-102

a. The ammonium nitrate concentration of the basal medium was varied. Cultures were followed for 9 days and sampled for ethylene and dry weight every 12 hours. The reported values are the maximum recorded during the 9 days. Time refers to maximum ethylene production with maximum dry weight occurring prior to this. Each value represents the average of four replicates of three flasks each.

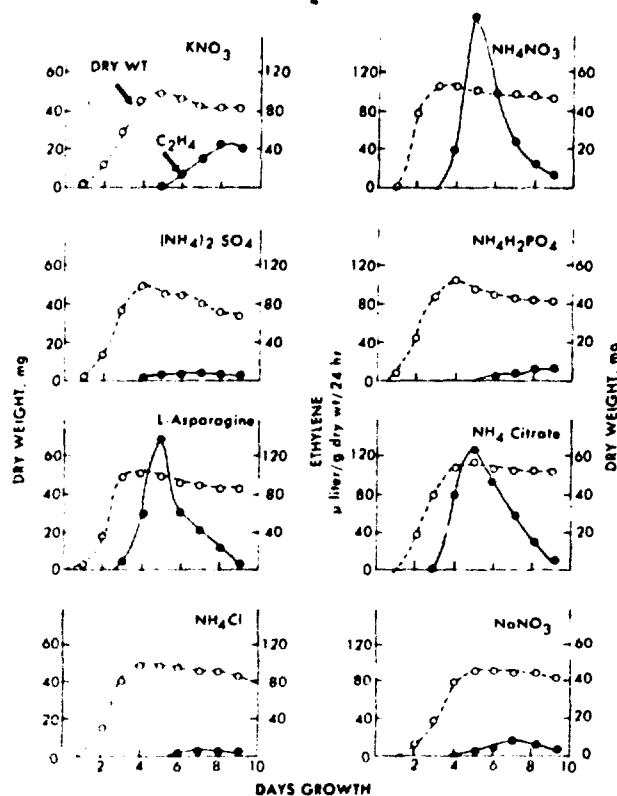


FIGURE 3. Effect of Various Nitrogen Sources on Growth and C_2H_4 Production. Each nitrogen source was substituted for the NH_4NO_3 in the basal medium to yield a nitrogen concentration of 1.4 g/liter. The cultures were grown at 25.2°C in the dark; different flasks were sampled every 24 hours for C_2H_4 and dry weight. Each value represents the average of four replicates of three flasks each.

F. EFFECT OF ETHANOL ON ETHYLENE PRODUCTION

Since Hall,¹⁰ Phan Chon Ton,¹¹ and Gibson¹² reported that feeding ethanol to *P. digitatum* resulted in a greater production of ethylene, this substrate for ethylene production by *P. cyclopium* was studied. Time-course studies of growth and ethylene production by *P. cyclopium* grown in the basal medium, with and without ethanol, were made for a 9-day period (Fig. 4). Four different flasks were stoppered for each 24-hour period. Maximum dry weights for the various ethanol treatments were: Control, 52 mg; 0.1%, 54 mg; 0.5%, 51 mg; 1.0%, 50 mg; and 3.0%, 58 mg. Except for the highest ethanol treatment, growth did not significantly increase with the addition of alcohol, but ethylene production did. In each treatment the ethylene production occurred after maximum mycelial growth had been reached. The greatest amount of ethylene was produced when the mycelial mats began to lose weight. Ethylene production was stimulated, but was not proportional to growth when ethanol was added to the basal medium.

G. EFFECT OF AN ALDEHYDE-FIXING AGENT ON ETHYLENE PRODUCTION

When ethanol was shown to have a stimulatory effect on ethylene production, studies were initiated to inhibit normal ethanol production in the fungus. Because no satisfactory method exists for trapping ethanol in vivo, several aldehyde-fixing agents were tested in an attempt to block the conversion of acetaldehyde to ethanol. Extensive work by Neuberg in the early 1900's as cited by Nord and Weiss¹¹ showed that sulfite and bisulfite salts were effective fixing agents of acetaldehyde. The use of other aldehyde-fixing agents, such as hydrazides, dimedone, and thiosemicarbazide, has the same effect as sulfite.¹²

Our experiments with the sulfite and bisulfite salts showed them to have an adverse effect on the growth of this fungus. Dimedone (5,5-dimethyl-1,3-cyclohexanedione) obtained from Eastman Kodak permitted good growth with no observable growth differences from cultures grown on the basal medium minus dimedone. The dimedone was sterilized with a Seitz filter and aseptically added to the medium before inoculation. Figure 5 shows the effect of dimedone on total ethylene production. Dimedone appears to be a good inhibitor of ethylene production in this fungus.

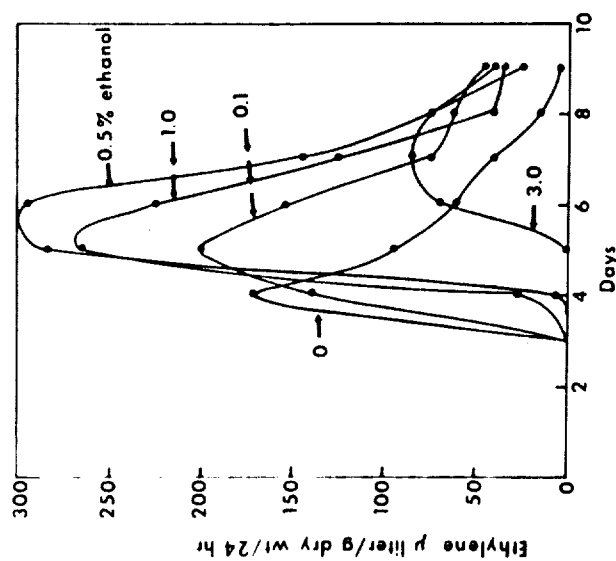


FIGURE 4. Effect of Ethanol on C_2H_4 Production. The ethanol was sterilized with a Seitz filter and added aseptically to sterile basal medium prior to inoculation. The cultures were grown 9 days at $25 \pm 2^\circ C$ in the dark. C_2H_4 and dry weight were measured every 24 hours from different flasks. Each value represents the average of four replicates of three flasks each. Total C_2H_4 , $\mu\text{liter/g dry weight}$, for 9 days was: Control, 395; 0.1%, 680; 0.5%, 850; 1.0%, 600; and 3.0%, 265.

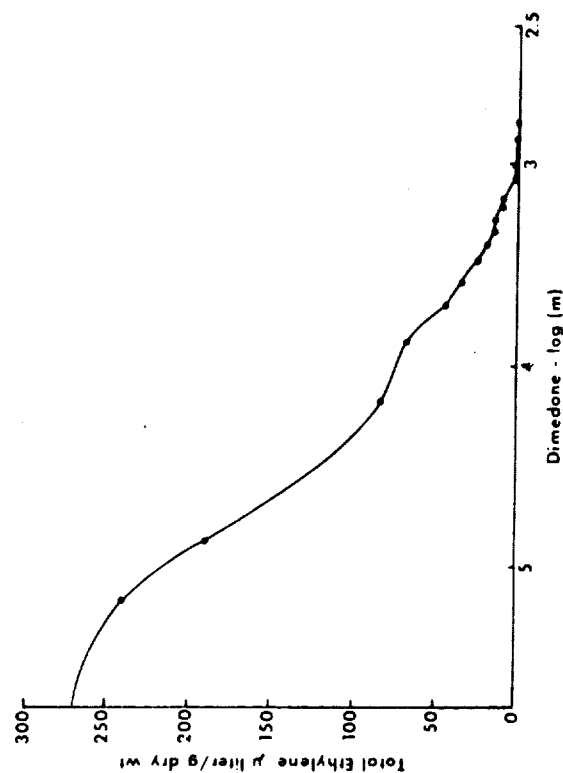


FIGURE 5. Effect of Dimedone on C_2H_4 Production. The dimedone was sterilized with a Seitz filter and added aseptically to sterile basal medium prior to inoculation. The cultures were grown for 9 days at $25 \pm 2^\circ C$ in the dark. C_2H_4 and dry weight were measured every 12 hours from different flasks. Total C_2H_4 refers to the amount produced during the 9 days. Each value represents the average of four replicates of three flasks each.

IV. DISCUSSION

The discovery that Penicillium cyclopium produced a gaseous metabolite chromatographically similar to ethylene prompted a more thorough characterization of the gas and the relationship between its production and the growth curve of the fungus. Preliminary experiments indicated that P. cyclopium grew well and produced ethylene on the basal medium and that growth was more uniform on solid than on liquid media. Heating the solid medium to liquefy the agar allowed determination of dry weights without difficulty.

Studies with P. digitatum have shown that ethylene is produced throughout the life cycle.^{15,13-18} However, it appears from some of the published data that although ethylene was produced continuously, the peak of ethylene production did not occur until after the dry weight reached a maximum.^{10,13,16} In contrast, P. cyclopium does not produce any ethylene (>0.05 ppm) until the dry weight reaches its maximum value. At the probable onset of autolysis the ethylene production increases rapidly to a peak and within 48 hours the ethylene production declines rapidly to a much lower level, where it remains for the duration of senescence.

Although a number of other fungi have been reported to produce ethylene, P. digitatum is the only fungus in which the structure of the gaseous emanation has been verified by conversion to chemical derivatives. Consequently, we converted the ethylene produced by P. cyclopium to 1,2-dibromoethane and compared its infrared spectrum with that of authentic 1,2-dibromoethane. The spectra were identical, thereby proving conclusively that P. cyclopium produces ethylene as a metabolic product.

Factors found to affect the growth of P. cyclopium did not always affect ethylene production the same way. When the sucrose concentration was varied, the ethylene produced was proportional to growth. Work with P. digitatum has shown that ethylene is closely associated with growth.¹⁶

When the source of nitrogen was changed or the concentration of ammonium nitrate varied, ethylene production was not proportional to growth in P. cyclopium. It has also been shown that abundant growth does not necessarily indicate high ethylene production in Penicillium digitatum.^{13,18}

Fergus¹⁷ reports that P. digitatum does not require any specific nitrogen source to produce ethylene. If organic and inorganic nitrogen compounds allowed growth, ethylene was produced. Figure 3 shows that P. cyclopium, when grown on various nitrogen compounds, always produced ethylene but the amount was dependent on the nitrogen source.

Ethylene production is shown to be affected not only by sucrose concentrations but possibly more significantly by nitrogen metabolism. Since ethylene production does not begin until the onset of autolysis in this fungus, it appears likely that the role of nitrogen is that of protein nitrogen during senescence. The respiratory quotient during ethylene production could reflect the metabolism of some proteins as well as fats.

Ethanol was found to stimulate ethylene production of P. digitatum by a number of workers.¹⁰⁻¹² Only a few of the many compounds tested have shown such a marked stimulation of ethylene in the Penicillia. Ethanol is an appealing precursor of ethylene.

Penicillia are ethanol producers and acetaldehyde may be found either as a precursor of ethanol or during its oxidation.¹³ Numerous other fungi have also been shown to produce ethanol both aerobically and anaerobically.¹⁴ Many of the fungi reported to produce ethylene have also been shown to be good alcohol producers. Among the highest ethanol producers is Aspergillus clavatus,¹⁵ which, interestingly, is reported to be the largest ethylene producer of the fungi examined by Ilag and Curtis.¹⁶

Because a specific inhibitor of ethanol synthesis is not available, an aldehyde-fixing agent was used. An experiment was carried out to fix the acetaldehyde, a primary precursor of ethanol in yeast fermentation, with dimedone. As an inhibitor dimedone does not appear to have any visible effects on the growth of the fungus, including the dry weight. As shown in Figure 5, dimedone, at a concentration of 10^{-4} M, inhibited 70% of the total ethylene production. Both ethanol (Fig. 4) and acetaldehyde (Fig. 5) have significant effects on ethylene production. Acetaldehyde, unlike ethanol, is a very poor precursor of ethylene.^{10, 15}

Figure 2, on the effect of inoculum size on growth and ethylene, shows a relationship similar to one observed when Penicillium notatum is grown for penicillin production. In general, conditions that favored the fastest growth of the fungus yielded the most penicillin. This requirement for fast and abundant growth also appears to yield the most ethylene in P. cyclopium. This may help to explain why (Table 2) the lowest and highest concentrations of ammonium nitrate yield significantly less ethylene, when the other concentrations have been shown to produce large quantities. This experiment indicates that inoculum size should not be overlooked when considering variability in ethylene production from fungi.

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